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A Tridecamer DNA Sequence Supports Human Mitochondrial RNA 3'-End Formation In Vitro

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Vertebrate mitochondrial genomes contain a putative transcription termination site at the boundary between the genes for 16S rRNA and leucyl-tRNA. We have described previously an in vitro transcription system from human cells with the capacity to generate RNA 3' ends with the same map positions as those synthesized in vivo. By assaying the ability of variously truncated templates to support 3'-end formation, we demonstrated that the tridecamer sequence 5'-TGGCAGAGCCCGG-3', contained entirely within the gene for leucyl-tRNA, is necessary to direct accurate termination. When two tridecamer sequences and their immediate flanking regions were placed in tandem, termination occurred at both promoter-proximal and promoter-distal sites. Furthermore, termination was competitively inhibited, in a concentration-dependent manner, by DNA containing the tridecamer sequence. These results suggest a modest sequence requirement for transcription termination that is contingent on a factor capable of recognizing the presence of the tridecamer DNA sequence.

Transcription proceeds in both directions from two divergent promoters located in the displacement loop (D loop) region of vertebrate mitochondrial DNA (mtDNA). The resulting polycistronic precursor RNAs are processed into mature transcripts by cleavages between the gene sequences (8). In vivo, the rRNA genes adjacent to the D loop region are transcribed 50 to 100 times more frequently than are the more distal heavy-strand genes; transcription termination has been proposed to account for this disparity (12). Transcripts initiated at the heavy-strand promoter (HSP) could either be elongated to encompass the entire heavy strand or be terminated at the 3' end of the 16S rRNA gene. We have described previously an in vitro system which terminates transcription at this site (7); we use termination as an economical description of RNA 3'-end formation at this location. The 3' ends of in vitro-terminated transcripts were mapped by S1 nuclease protection 3 and 4 nucleotides (nt) upstream of the 16S rRNA-tRNA^{Leu} gene boundary, in agreement with in vivo data. All DNA sequence information necessary for in vitro termination appeared to reside in a 151-base-pair (bp) fragment centered around the gene boundary.

In this study we present evidence that a short DNA sequence supports termination at the 3' end of the human mitochondrial 16S rRNA gene. Furthermore, competition experiments suggest that a titratable factor is involved in termination. Finally, we observed other sites of RNA 3'-end formation in vitro which mapped at mtDNA sequences bearing similarities to the 16S rRNA-tRNA^{Leu} boundary; the exact nature and any physiological significance of these remain to be determined.

MATERIALS AND METHODS

Plasmid construction. The human light-strand promoter (LSP) sequences were obtained either from clone L5'Δ-33 or from clone L5'Δ-28 (4). Human sequence from nt 585 to 741 came from clone L3'Δ-178. A *Hae*II human mtDNA fragment cloned in pACYC177 (19) supplied nt 3160 to 4534. DNA sequences and nucleotide numbering for human mtDNA throughout are taken from Anderson et al. (1). Clone A consists of nt 445 to 324 (containing the LSP), a 10-bp *Eco*RI linker, and nt 3165 to 4121 (containing the termination site), all inserted between the *Bam*HI and *Eco*RI sites of pBR322. Clone B is the same as clone A except that the LSP is abbreviated by 10 bp, thus extending from nt 435 to 324; clone B was also used in our earlier study (7). Clone C consists of nt 445 to 324 joined to an 8-bp *Sal*I linker inserted between the *Bam*HI and *Sal*I sites of M13mp18. Clone D has a 15-nt oligonucleotide, 5'-TCGACTGG CACTGCA-3', inserted between the *Sal*I and *Pst*I sites of clone C (9). Clone E consists of nt 445 to 1 inserted between the *Bam*HI and *Eco*RI sites of pBR322. Clone F consists of nt 585 to 741, a 10-bp *Eco*RI linker, and nt 3165 to 4121 inserted between the *Bam*HI and *Eco*RI sites of pBR322. Clone G contains two termination sites in tandem; it consists of nt 435 to 324, a 10-bp *Eco*RI linker, nt 3165 to 3316, a 10-bp *Hin*dIII linker, and nt 3165 to 4121, all inserted between the *Bam*HI and *Eco*RI sites of pBR322.

Deletion mutagenesis. A series of 5' deletions extending into the termination site at the 16S rRNA-tRNA^{Leu} gene boundary was made by BAL 31 digestion (14). The resulting clones consisted of nt 435 to 324, a 10-bp *Hin*dIII linker, and a fragment whose length varied for the different clones, extending toward nt 3316. These fragments were inserted between the *Bam*HI and *Aat*II sites of pBR322. The 122 clones were first screened by *Hpa*II digests and 5% polyacrylamide gel electrophoresis. Those clones with deletions extending past the *Hpa*II site at nt 3246 were rescreened by *Hph*I digestion and were analyzed by agarose gel electrophoresis. From the 122 clones, 62 were selected for DNA sequencing (16). Ultimately, 29 unique clones were obtained.

A BAL 31-derived series of 3'-deleted clones was also constructed. These clones consisted of nt 435 to 324, a 10-bp *Eco*RI linker, a fragment starting with 3165 and ending at the deletion breakpoint, and a *Hin*dIII linker. These fragments

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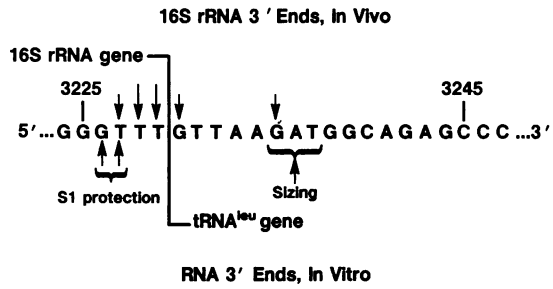

FIG. 1. The DNA sequence at the termination site. The arrows mark the 3' ends of RNA molecules from both in vivo and in vitro sources. For in vivo 16S rRNAs, the two longer arrows indicate the 3' ends of >25% of the molecules (10). The 3' ends of in vitro transcription products, as determined by S1 protection analysis and by sizing, are shown. As determined by sizing, the actual 3' end is at only one of the bracketed bases but uncertainty prevented an exact determination (7).

were inserted between the *Bam*HI and *Hin*dIII sites of M13mp18. Phages from 120 plaques were grown and analyzed by dideoxy DNA sequencing (18). After the 120 clones were sequenced, 11 unique clones in the region of greatest interest were selected.

Purification of transcription and termination activities. Mitochondrial RNA polymerase was prepared from human KB cell mitochondria and utilized as described previously (22), except that each fraction was dialyzed rather than just the peak fraction of polymerase activity. All transcription termination reactions were performed in a 50-μl volume containing 10 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiotreitol, bovine serum albumin (100 μg/ml), 180 μM CTP and UTP, 360 μM ATP, 0.01 μM [α-^{32}P]GTP (800 Ci/mol), 10 to 12 μl of heparin-Sepharose fractions, and, typically, 0.5 μg of DNA (10 μg/ml). The reactions were incubated at 15°C for 45 min, terminated by the addition of 10 μl of 200 mM EDTA, and extracted with phenol and chloroform. The products of the reactions were analyzed by electrophoresis in 1-mm-thick 7 M urea–8% polyacrylamide gels. In competition experiments, competing DNA was added in the form of either circular plasmid or gel-purified DNA fragment in amounts ranging from concentrations equimolar with the template DNA to six times the template DNA concentration.

RESULTS

The DNA sequence information specifying termination at the 3' end of the 16S rRNA gene (Fig. 1) resides within a 151-bp region extending from the 65 bp preceding the 16S rRNA-tRNA^{Leu} gene boundary to the 86 bp following the gene boundary (7). In order to determine which part of this region is essential for termination, a series of deletion clones was constructed by BAL 31 digestion. The deletions extended for various lengths from the ends of the original 151-bp termination fragment; in these constructions, the promoter was unaffected.

5'-Deletion analysis of termination region. Twenty-nine 5'-deletion clones distributed around the 16S rRNA-tRNA^{Leu} gene boundary were used as templates for in vitro transcription. The products of 18 of these reactions are shown in Fig. 2. The deletion templates were cleaved with the restriction enzyme *Nco*I; full-length runoff transcripts to this site were also synthesized. Both runoff transcripts and terminated transcripts became progressively shorter as the

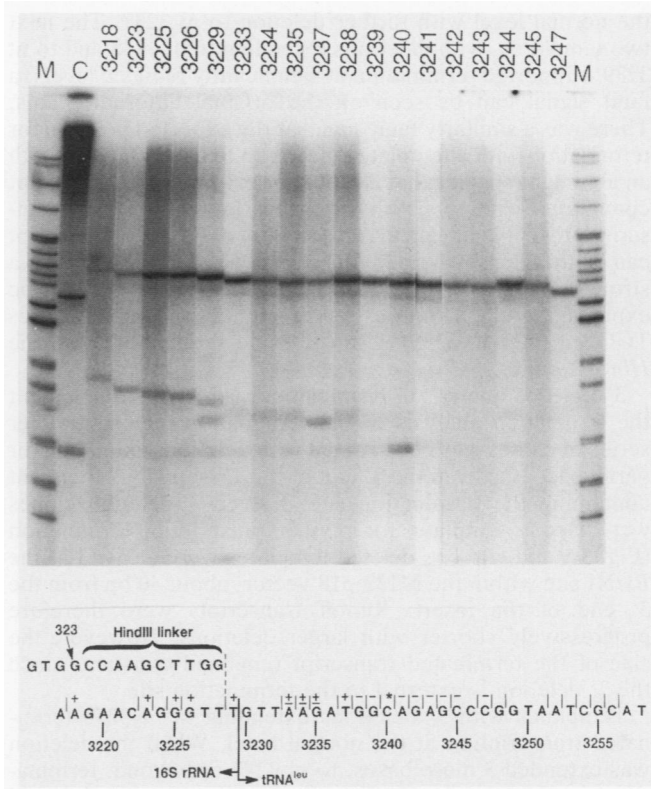

FIG. 2. Transcription termination reactions on a series of 5'-deleted clones. Plasmid clones with deletions extending into the termination region from the 5' direction were constructed as described in Materials and Methods. After cleavage with *Nco*I, 18 of these clones were used as templates for in vitro transcription termination reactions. The products were electrophoresed in lanes 3218 through 3247, with the numerical labels indicating the first base retained in a given deletion clone. An 84-nt-long transcript (discussed in the text) can be seen in all experimental reaction lanes. Lanes M contain denatured 5'-end-labeled *Hpa*II fragments of pBR322 as size markers. Lane C contains a control reaction with undeleted template; since the control template is not cleaved with a restriction enzyme, no runoff transcript is present and a high-molecular-weight smear is present where transcription past the termination site finally ends. The diagram at the bottom summarizes the termination data. Templates supporting termination (+), templates supporting little or no termination (−), and templates supporting reduced termination (±) are indicated. The asterisk marks the template deleted up to nt 3240, which has an apparent termination transcript of anomalous length (see text). The various templates have deletions extending to the short vertical lines above the sequence. As an example, one of these lines is extended upwards with a dashed line to show the intruding sequence. The numbers below mark the nucleotide sequence positions. Transcription is from left to right.

deletion extended further, because the deletion is within the transcribed region. Templates with deletions extending to the last nucleotide of the 16S rRNA gene (nt 3229) supported a normal level of transcription termination. Termination was absent in templates with deletions extending beyond nt 3240 (11 bases downstream of the 3' end of the 16S rRNA gene, within the tRNA^{Leu} gene).

Between nt 3229 and 3240, some deletions affected termination. A template deleted to nt 3229 supported normal termination and also the synthesis of an additional transcript 6 nt shorter. Deletions to nt 3233, 3234, and 3235 reduced the level of termination. Surprisingly, termination returned to

the normal level with further deletion to nt 3237. The next two templates, with deletions extending to nt 3238 and to nt 3239, supported termination at significantly reduced levels; a faint signal can be seen on the original autoradiograms. There was a similarly faint signal at the expected position for termination with a template deleted up to nt 3240, along with an abundant species that is 6 nt shorter. The latter transcript comigrates with, or is an intensification of, an 84-nt transcript present in all lanes. A discussion of the 84-nt transcript can be found elsewhere in this paper. The reappearance of a strong band with the template deleted to nt 3240 might be explained by the fortuitous replacement of the deleted bases TGG (nt 3237 to 3239) with the same sequence within the *Hin*dIII linker.

3′-Deletion analysis of termination region. To complement the 5′-deletion analysis of the termination region, another series of clones was constructed with deletions extending for various lengths from the 3′ end of the 151-bp DNA fragment containing the termination site. Eleven 3′-deletion clones were used as templates for in vitro transcription termination (Fig. 3A and C). The deleted templates were cleaved at the *Bst*NI site within the M13mp18 vector, about 40 bp from the 3′ end of the insert. Runoff transcripts were therefore progressively shorter with larger deletions. However, the size of the terminated transcript remained constant, since the 3′ deletion is external to the termination site.

Templates with 3′ deletions extending to nt 3250 terminated transcription at the normal level. When the deletion was extended 3 more bases, to nt 3247 or beyond, termination no longer occurred. From these data it appeared that 3′ deletions abolished termination more abruptly than did 5′ deletions, which exhibited variable effects before longer deletions eliminated termination. Taken together, the 5′- and 3′-deletion data showed that sequences important for termination are located between nt 3229 and 3249; the essential region is the tridecamer sequence 5′-TGGCAGAGCCCGG-3′ from nt 3237 to 3249.

Termination at other sites. In another experiment, the same series of 3′-deleted templates was transcribed in reactions with at least twice as much DNA (20 μg/ml rather than 10 μg/ml). Termination (or lack thereof) at the usual site was unaffected, but other transcripts appeared (Fig. 3B). The 3′ ends of these transcripts mapped by length near the junction between the *Hin*dIII linker and the M13mp18 DNA in the encroaching deletions (Fig. 3C and Fig. 4, line 2).

While the DNA sequence at the linker-M13 junction apparently has an intrinsic ability to specify termination, its efficacy is enhanced by 3′ deletions extending to nt 3247 through 3238. Presumably, deletions that extend past these positions delete DNA sequences at the 16S rRNA-tRNA^{Leu} mitochondrial termination site that would otherwise help to specify termination. Short deletions may have the linker-M13 junction too far from these mitochondrial termination sequences, as well as perhaps having to compete with standard termination at the gene boundary.

A pentanucleotide sequence, TGGCA, is found both at the 16S rRNA-tRNA^{Leu} gene boundary termination site and at the *Hin*dIII linker-M13mp18 junction. To test whether this pentanucleotide alone is sufficient for termination, a 15-mer oligonucleotide comprising the pentanucleotide flanked by *Sal*I and *Pst*I cohesive ends was synthesized and inserted downstream of the LSP fragment in clone C to make clone D. When clone D was used as a template for in vitro transcription termination, no terminated transcripts were produced (data not shown). Therefore, while the sequence TGGCA could be critical in termination, it is likely insufficient to specify a termination event. This agrees with the deletion analyses indicating that as many as 13 bases are involved in termination.

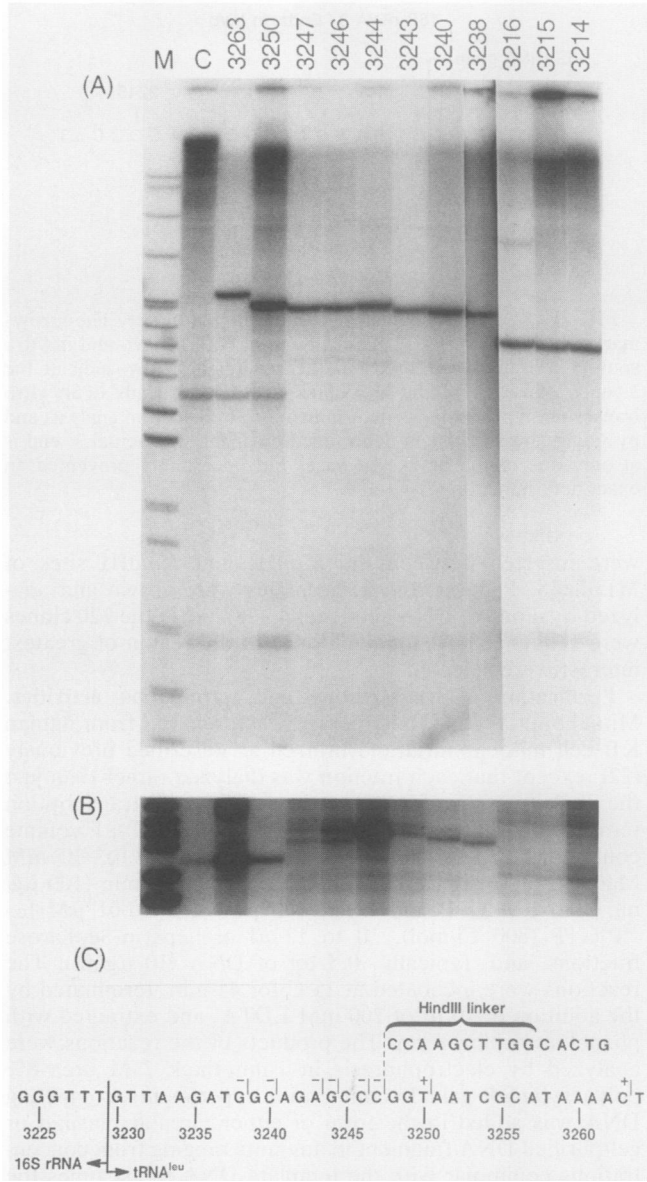

FIG. 3. Transcription termination reactions on a series of 3′-deleted clones. M13mp18 clones with deletions extending into the termination region from the 3′ direction were constructed as described in Materials and Methods. Replicative-form DNAs from these clones were cleaved with *Bst*NI and used as templates for in vitro transcription termination reactions. These were electrophoresed in lanes 3263 through 3214 (the numbers indicate the first 3′ base retained in the deletions of the termination region) with 10 μg of DNA per ml as the template (A) and 20 μg of DNA per ml as the template (B) (lanes 3263 and 3244 in panel B, 40 μg of DNA per ml as the template). Lanes M and C are the same as in Fig. 2. The 84-nt transcript in all experimental reaction lanes is discussed in the text. (C) Summary of termination data; the symbols and labels are the same as in Fig 2. Transcription is from left to right.

An 84-nt transcript, which may be a terminated product, was seen in Figs. 2 and 3. The 3′ end of this transcript mapped by length to nt 324 at the junction between the mtDNA D loop sequence and the *Eco*RI (or *Hin*dIII) linker used in constructing these cloned templates. The sequences

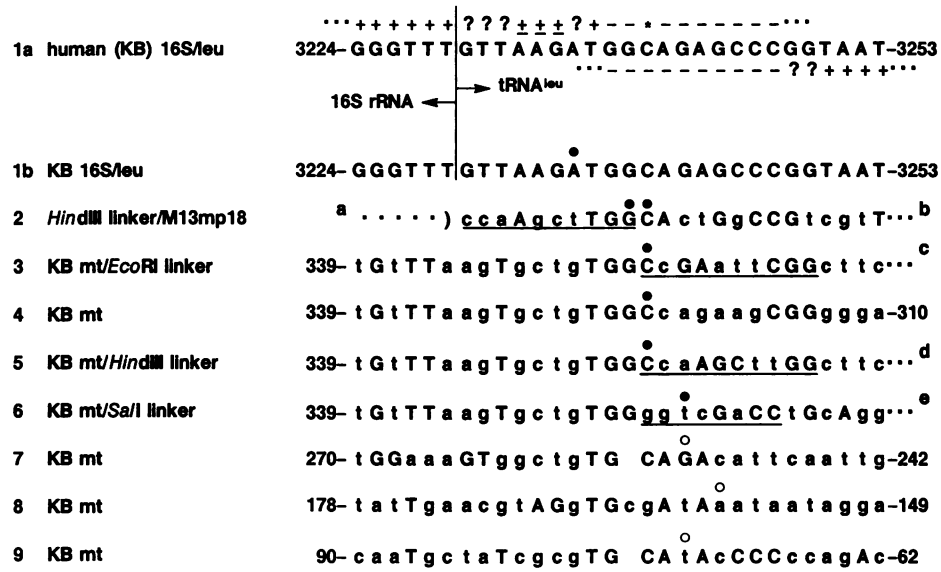

FIG. 4. Sequences at in vitro termination sites. Line 1a summarizes the information from Fig. 2 and 3. Above and below the sequence are indicated, respectively, the effects on termination of 5′ and 3′ deletions. Question marks are used where it is not clear whether termination would occur in templates deleted to that base; deletions to these nucleotides were not found. The asterisk is used as in Fig. 2. In lines 1b through 9, the 3′ end of the in vitro transcript, as measured by length against the DNA sequence, is indicated (● [precise] or ○ [estimated]). Capital letters indicate positions in each sequence which are common with the termination site after the 16S rRNA gene; for lines 7 and 9, a single-base-pair adjustment has been allowed in the alignment. Some template constructions have linkers, which are underlined. The meanings of the superscripts (a through e) are as follows (all superscripts on the right indicate bases which are not mitochondrial in origin): a, dots and parenthesis indicate that different DNA sequences fill this position in the various 3′ deletions; b, all base pairs shown, *Hin*dIII linker joined to M13mp18 sequence; c, last 14 bp, *Eco*RI linker joined to pBR322 sequences; d, last 14 bp, *Hin*dIII linker joined to pBR322 sequences; e, last 14 bp, *Sal*I linker joined to M13mp18 sequences.

created at both junctions are shown in Fig. 4, lines 3 and 5. Although the intensity of the 84-nt transcript varied relative to the normal termination transcript, differences in DNA concentration do not account for the variation. Four of the five bases of the pentanucleotide discussed above (TGGC) are found at the site of apparent termination for the 84-nt transcript.

The apparent termination site at nt 324 could be due exclusively to the mitochondrial sequences at this site retained in the clones or to a fortuitous combination of mitochondrial and linker sequences. Most of the cloned templates constructed for the experiments in this paper joined a linker to nt 324, which during cloning is exposed as a blunt end by *Bal*I restriction cleavage of the mitochondrial sequence containing the LSP. Another clone was constructed without *Bal*I cleavage or linker addition which included an additional 324 bp of the D loop region downstream of the LSP (clone E) (Fig. 5). The 84-nt transcript also appeared in transcription termination reactions with this template (Fig. 5, lane 1). It should be noted that this transcript was too faint to be seen in the control lane (Fig. 5, lane 2), although it is present in original autoradiograms. This experiment demonstrated that the addition of a linker at nt 324 is not necessary to form a termination sequence; either the mitochondrial sequence upstream from nt 324 is sufficient or the linker fortuitously replaces a few critical base pairs downstream from nt 324 (Fig. 4, lines 3 to 5). This was tested by the addition of an 8-bp *Sal*I linker and M13mp18 sequences rather than the *Eco*RI or *Hin*dIII linkers, causing a different sequence change (Fig. 4, line 6). In this experiment, the 84-nt transcript was replaced by an 86-nt transcript (data not shown). As can be seen in Fig. 4, lines 3 to 6, both the mitochondrial sequence and the linker-modified sequences are similar to the known termination site at the 16S rRNA-tRNA^{Leu} gene boundary. Most of the common bases are clustered at the two ends of this sequence, about one turn of the DNA helix apart.

The 3′ end of the 84-nt transcript is only a few base pairs closer to the LSP than is an RNA-processing site associated with a conserved sequence block (CSB II) previously identified by in vitro cleavage (6). The connection, if any, between these two events is unclear. Besides the 84-nt transcript, there are a number of other species in lane 1 of Fig. 5. One of these transcripts is 93 nt long and maps to mtDNA nt 315. This site is within CSB II, 2 bp downstream from the in vitro processing site mentioned above (6), and is 6 bp from a mapped 3′ end of an in vivo transcript (5); it may be analogous to either transcript in this sizing range. There are three abundant, larger transcripts (besides the 416-nt runoff) whose lengths were estimated to be 155, 250, and 330 nt. By transcript length, corresponding RNA 3′ ends would fall around mitochondrial nt 253, 158, and 78. These transcripts may result from termination, because the distal fragments which could arise through processing are absent; however, RNA processing cannot be excluded. All three of these sites have a short identity with the known termination site at the 16S rRNA-tRNA^{Leu} gene boundary (Fig. 4, lines 7 to 9).

Tandem termination. If the transcription complex is committed to termination during initiation, templates with tandem termination sites might be expected to exhibit termination only, or at least preferentially, at the promoter-proximal site. To test this hypothesis, clone G was constructed with a reiterated 151-bp fragment containing the termination site downstream of the fragment containing the LSP. This uncleaved plasmid was used as a template for in vitro transcription (Fig. 6). Besides the 167-nt transcript also observed in the control (Fig. 6, lane 1), another transcript was seen (Fig.

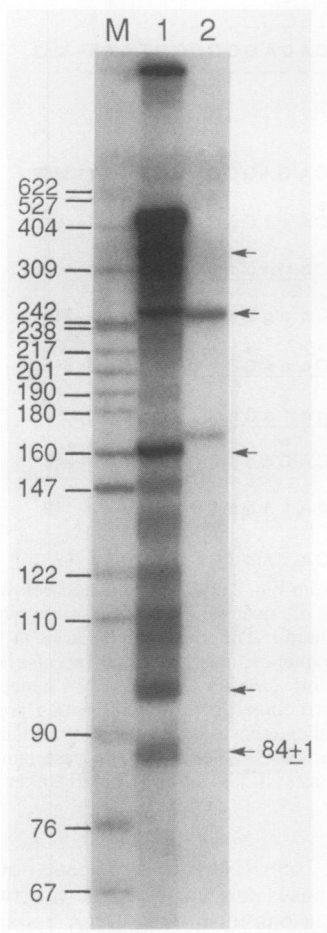

FIG. 5. Transcription termination in the D loop region. Clone E contains nt 1 through 445 followed by an *Eco*RI linker after nt 1. Distal to the LSP are 407 bp of D loop-derived sequence. The plasmid was digested with *Eco*RI and used as a template for in vitro transcription. The products were electrophoresed in lane 1. Five transcripts in this lane are marked with arrows. The top band, not marked, is the 416-nt runoff transcript. In lane 2, in vitro transcription termination products from *Bal*I-cleared clone A were used for a control. The termination (167 ± 1 nt) and runoff (246 ± 1 nt) transcripts are visible; the 84 ± 1 transcript is also present on the autoradiogram in lane 2. For size markers, an *Hpa*II digest of pBR322 was electrophoresed in lane M. Numbers on the left indicate nucleotides.

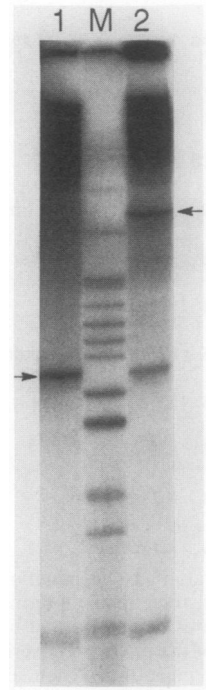

FIG. 6. Tandem termination. A template, clone G, was constructed with a duplicated 151-bp fragment containing the termination site at the 16S rRNA-tRNA^{Leu} gene boundary. The products of in vitro transcription on this uncleaved template were electrophoresed in lane 2, and the products transcribed from uncleaved clone B were electrophoresed in lane 1 as a control. The arrow on the left indicates the 167-nt termination transcript common to both templates. The arrow on the right marks the 328-nt transcript from the duplicated termination site. For size markers, an *Hpa*II digest of pBR322 was electrophoresed in lane M.

6, lane 2). If the second termination site were functional, a 328-nt transcript would be present; this is the length of the longer transcript in Fig. 6, lane 2 (arrow). The equal utilization of tandem termination signals, together with the fact that both the HSP and LSP support transcriptional termination at the 16S rRNA-tRNA^{Leu} gene boundary (7), argue against a critical role for the promoter in transcriptional termination in vitro.

DNA competition affects mitochondrial termination. To test for the possible presence of a factor that might interact with the mtDNA sequence at the termination site, competing DNA was added to transcription termination reactions containing clone A cleaved with *Bal*I (Fig. 7). The template was cleaved so that competition effects on both transcription and termination could be assessed. In one series, clone F was added as a competitor. Clone F contains sequences downstream of the HSP (nt 585 to 741) as well as nt 3165 to 4121 containing the termination site. This clone is therefore analogous to template clone A but without a mitochondrial promoter. The competing DNA was added to reactions at concentrations one, two, or four times that of the promoter-containing template, and the products were electrophoresed adjacent to those of a control incubation of promoter-containing template without competitor (Fig. 7, lanes F to I). The competing DNA was effective in reducing termination; in fourfold excess, transcription itself was reduced, while it was enhanced at lower concentrations of competitor. To distinguish a general carrier DNA effect from a specific influence of the termination region, a fourfold excess of pBR322 was added to one reaction (Fig. 7, lane J). This addition enhanced transcription (a result for which we currently have no explanation) but caused only a modest reduction in termination. A mixture of nonspecific and specific competitor DNA (Fig. 7, lane K) had an effect equivalent to that of the same amount of specific competitor (Fig. 7, lane H). These results indicate that a plasmid containing the termination site is a more effective competitor of transcription termination than a plasmid without the termination site.

In another series of experiments, three isolated DNA fragments were used as competitors. A 152-bp *Eco*RI-*Bal*I fragment (nt 3165 to 3316) containing the termination site was used as the termination-plus competitor. A 273-bp *Bal*I-*Eco*RI fragment from elsewhere in the human mitochondrial genome (nt 3851 to 4123) and a 358-bp *Sau*3AI fragment from pBR322 were used as termination-minus competitors. The 152-bp termination-plus competitor DNA

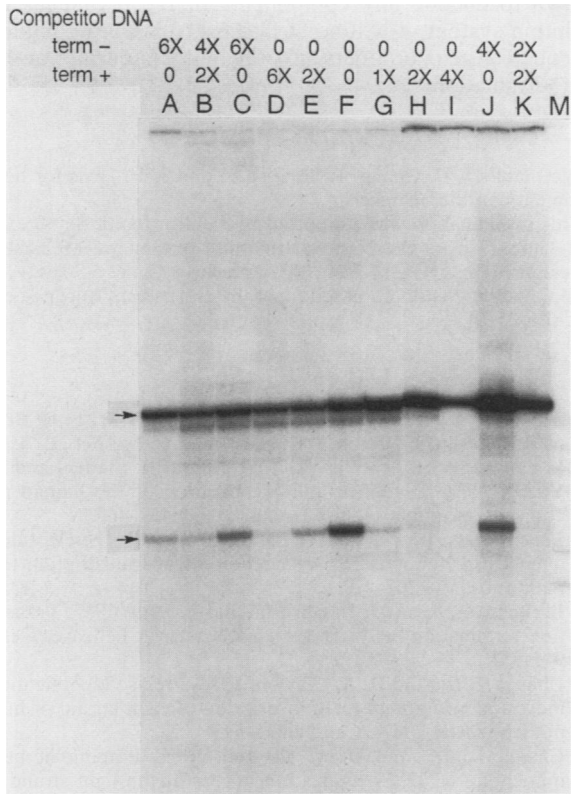

FIG. 7. Influence of competitor DNA on transcription termination. For all lanes, 0.5 μg of *Bal*I-cleaved clone A was used as template for a standard transcription termination reaction. Competitor DNA was also added to the reactions for all the lanes except control lane F. Lane F therefore exhibits normal runoff (upper arrow) and terminated (lower arrow) transcripts. In lanes G through K, various amounts of closed circular plasmid DNA were added as competitors. The amount of competitor relative to the template is given above the lanes; 1× is 0.5 μg of competitor DNA. Competing plasmid with the termination site (term +) was clone F, while the plasmid without the termination site (term −) was pBR322. In lanes A through E, various amounts of gel-purified DNA fragments were added as competitors, and the amounts of competitor fragments were multiples of the molar quantities of template DNAs. The termination-plus fragment was 152 bp in length and was added in the stated amounts to lanes B, D, and E. A 273-bp termination-minus fragment was used in lanes B and C, while a 358-bp termination-minus fragment was used in lane A.

in twofold molar excess over promoter-containing template reduced termination relative to the competitor-free control and in sixfold molar excess reduced it even more (Fig. 7, lanes D to F). Both the 275-bp and 358-bp termination-minus competitors had an effect on termination when present in sixfold molar excess over promoter-containing template (Fig. 7, lanes A and C), but this effect was significantly less than the effect of the same amount of termination-plus competitor. A mixture of the 152-bp fragment (in twofold excess) and the 273-bp fragment (in fourfold excess) reduced termination more effectively (Fig. 7, lane B) than did the 152-bp fragment in twofold excess alone (Fig. 7, lane E) and much more effectively than did a sixfold excess of the 273-bp termination-minus competitor fragment, a difference present but less pronounced compared with the 358-bp fragment. Although the termination-minus competitor fragments did not contain the tridecamer termination site, a range of incomplete identities was present in the 358-bp fragment which might have resulted in the limited competition observed.

In general, whether present as whole plasmid or as an isolated fragment, competitor DNA containing the termination site was more effective in reducing termination in vitro than was DNA without the termination site. This suggests that DNA at the termination site may interact with and sequester a factor(s) required for transcriptional termination.

DISCUSSION

Deletion analyses define a sequence sponsoring RNA 3′-end formation. A major conclusion of this work is that the human mtDNA tridecamer template sequence 5′-TGGCAGAG CCCGG-3′ is effective in supporting RNA 3′-end formation. Encroachment from either the 5′ or the 3′ direction into this sequence results in a drastic drop in termination efficiency. We note that the termination region functions bidirectionally, at least in vitro (7), and we have not tested the ability of the various truncated templates to abet termination of polarity opposite that of 16S rRNA synthesis.

It is interesting that the tridecamer termination sequence is contained entirely within the 5′ end of the gene for leucyl-tRNA; this species, in contrast to other mitochondrial tRNAs, possesses the usual invariant features of tRNAs and shows a high degree of conservation among vertebrates. In fact, the tridecamer sequence itself is even more highly conserved (e.g., 100% similarity between human and bovine tridecamers and 92% similarity between human and mouse tridecamers). This may be explained by the dual functional constraints, encoding a tRNA and directing transcription termination, placed on this portion of the genome.

Additional cases of in vitro RNA 3′-end formation, mapping to both mtDNA and vector template positions, were observed; in the case of either template, certain sequence elements were common with the tridecamer terminator sequence (Fig. 4). The proximity of some of the putative termination sites with identified RNA-processing sites in the D loop region (5, 6) is suggestive. We note that at least two modes of RNA-end formation, processing of a primary transcript to produce $tRNA^{Leu}$ and rRNA termination, are occurring at the 16S rRNA-$tRNA^{Leu}$ gene boundary in vivo (20).

Termination versus processing. It is widely appreciated that efficient RNA processing could mimic a termination event. We have used termination to describe the process by which RNA 3′ ends are generated in the reactions described here and in previously described reactions (7). This conclusion was drawn mostly on the basis of two lines of evidence. First, processing cleavage in runoff reactions could yield specific 3′ fragments as well as the observed 5′ fragments, but 3′ fragments were absent. (However, there is precedent for the lack of detection of 3′-distal portions in processed eucaryotic mRNA populations.) Also, processing cleavage in runoff reactions should alter the ratio of 5′ fragments to whole runoff transcripts with time, but incubation times do not affect this ratio (unpublished observations). Second, isolated runoff transcripts were not processed when incubated in the reaction conditions without a template (7). The competition experiment reported in this paper provides a third line of evidence for termination rather than processing. When competing DNA containing the terminator sequence was added to the transcription termination reaction, the amount of terminated transcript was reduced. Processing cleavage of an RNA transcript should likely not be inhibited by a sequence-specific, double-stranded DNA competitor.

Purification of obligatory factors in addition to the template is required to resolve the mechanism of 3'-end formation.

What are the requirements for termination? A number of mechanisms can specify transcription termination. A factor binding to the nascent RNA transcript can induce termination, as in the case of rho-dependent termination in *Escherichia coli* (3, 21). The nascent RNA transcript alone can cause termination by folding into a secondary structure specified by the DNA sequence near the termination site, as happens in rho-independent termination (21). The polymerase could respond to a terminator DNA sequence either as core polymerase (2) or with the assistance of an associated termination factor. Finally, a factor could recognize and bind to the DNA at or near the termination site and direct transcription termination, as may be the case for mouse nuclear 28S rRNA (13).

A model for termination involving an RNA-binding factor in mitochondria, analogous to rho-dependent termination, is not supported by experimental data; any role of specific nascent RNA in termination is eliminated by the 5'-deletion analysis. Deletions which extend down to and past the 3' end of the natural transcript still permit termination. Furthermore, the termination activity can be competed for by DNA containing the termination sequence, and the termination site is bidirectional. Neither of these effects would be expected from a rho-dependent type of termination.

The structural similarity of the 3' end of the mitochondrial 16S rRNA to a rho-independent termination site has been pointed out (10). A model without polymerase-associated factor requirements is modestly attractive, given that core mitochondrial RNA polymerase may in part function as does bacteriophage T7 RNA polymerase (15). In this regard, it is known that T7 RNA polymerase is a single polypeptide that terminates transcription at a specific DNA sequence, with no known demand for additional factors (11). However, the 5' deletions again rule out any role for specific nascent RNA sequences; the effects of competing DNA and the bidirectionality of the termination site also contradict a rho-independent type of termination.

A model providing for recognition of the tridecamer sequence itself by the RNA polymerase is not eliminated by the deletion analysis. However, the experiment with competing DNA argues against simple sequence recognition by the RNA polymerase. Furthermore, to be effective in bidirectional termination with this model, the polymerase transcribing the complementary strand would likely need to recognize the same short termination sequence. This would require a palindromic sequence, which is not present.

Regardless of the complexity of the reaction, several basic features can be excluded. DNA (or RNA) sequences flanking the very short terminator are not critical to termination. Contrary to some previous predictions (17), the nature of the mtDNA promoter sequence involved appears not to be important, at least in vitro. Consistent with the lack of a specific promoter requirement is the apparent absence of a transcription complex commitment to termination (Fig. 6).

The most interesting possibility, consistent with all of the above arguments, emerged from the experiment regarding template competition for termination. Here it was revealed that competing termination sequence reduced termination from a coincidentally added active template. The simplest interpretation of this observation is that the termination sequence can sequester a critical component. An affinity approach may permit identification and isolation of an ancillary component(s) directing transcription termination. Because mitochondrial genomes from yeast to man are not known to encode any components of the organellar transcription system, it will be of interest to learn the nature of any nuclear gene products affecting mitochondrial transcription termination.

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